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Laser-stimulated fluorescence of submicrometer regions within single mitochondria of rhodamine-treated myocardial cells in culture

(heart/contractility/microscopy/spectroscopy)

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ABSTRACT **A focused laser beam of 442 nm was used to stimulate fluorescence in 0.5-μm spots in single mitochondria of myocardial and endothelial cells in culture. Cells were treated with rhodamine 6G or 123 in order to render the mitochondria fluorescent. Rhodamine 123-treated cells exhibited a gradual decrease in fluorescence over several minutes, whereas the rhodamine 6G-treated myocardial cells exhibited three distinct patterns of variable fluorescence intensity. These patterns were detected at different points within the same mitochondrion or in different mitochondria. Mitochondria from nonmyocardial endothelial cells did not exhibit any variable intensity patterns of fluorescence. Electron microscopy revealed no ultrastructural damage attributable to laser exposure of the mitochondria. The variable fluorescence patterns are hypothesized to be indicative of localized alterations in molecules or ions at the suborganelle level.**

Most studies on the physiology of mitochondria either are conducted on extracted mitochondrial preparations (1, 2) or they are static studies in which mitochondrial ultrastructure is examined (3, 4). Little, if any, work has been done on the living cell because *in situ* studies on a specific class of cellular organelles are technically very difficult. Experimental studies on only one organelle, let alone subregions within one organelle in a living normally functioning cell, have been virtually impossible to do.

We report here the use of a focused laser beam to stimulate localized fluorescence in submicrometer regions of single mitochondria in contracting myocardial cells in culture. The mitochondria have been photosensitized by treatment with rhodamine 6G so that fluorescence can be stimulated with 442-nm laser light. Changes in rhodamine fluorescence have been shown to be associated with charge separation in mitochondria (5) and surface potential and intramembrane potential changes in *Tetrahymena* (6).

In the system reported here, the myocardial ventricle cells continue to contract rhythmically up to 48 hr after rhodamine treatment and appear to be physiologically normal. By focusing a laser to a 0.5-μm spot inside single large (2–4 μm) mitochondria it has been possible to stimulate and record fluorescence from multiple sites within the same organelle while the cell continues to contract. This method permits the detection of localized distributions and oscillations of molecules and ions within a single organelle in a live cell.

MATERIALS AND METHODS

Cell Culture. Cultures of rat myocardial cells were established from ventricles of 2- to 4-day old rats by using standard procedures described in earlier studies (7, 8). The cultures were established in Rose tissue culture chambers on no. 1 thickness coverglasses. Cells were grown in a standard minimal essential culture medium fortified with antibiotics and with phenol red as a pH indicator. Cultures were maintained at 37°C for 3–4 days prior to use in the experiments. By day 3 all the cells had flattened out on the coverglass and three general classes of cells could be distinguished: contracting myocardial cells, noncontracting myocardial cells, and nonmuscle endothelial cells. All three cell types were selected for laser irradiation.

Fluorescence Photosensitization. Aqueous stock solutions (1 mg/ml) of both rhodamine 6G and rhodamine 123 were diluted into heart medium to yield final dye concentrations of 1.8 and 2.6 μM for rhodamines 6G and 123, respectively. These solutions were then injected into Rose chambers containing the heart cell cultures. The cells were exposed to the rhodamine solutions for 30 min and rinsed two times for 10 min with fresh culture medium. One hour after rhodamine exposure the cells were examined under a standard Zeiss RA epifluorescence microscope using a 50-W mercury lamp as a light source. Epifluorescent filter sets Zeiss no. 437715 or no. 487709 were used. These filter sets permitted observation of the fluorescent patterns of the entire cell. Cells were observed by fluorescent microscopy 1, 5, 24, and 48 hr after treatment with the rhodamines. Photomicrographs were made by using a standard 35-mm camera mounted on the microscope with Kodak Tri-X film exposed for 1–2 min and processed with Acufine developer, pushing the ASA to 800.

Laser Microirradiation and Fluorescence Detection. A system similar to that used in membrane photobleaching studies was employed (9). A continuous wave (CW) helium/cadmium (He/Cd) laser at wavelength 442 nm (output 6–8 mW) was directed into the normal mercury lamp attachment port of a standard epifluorescent Zeiss RA microscope. Prior to entry into the microscope, the laser beam passed through a spatial filter, a recollimating lens, and a set of neutral density filters. The neutral density filters were used to attenuate the laser beam to levels that were high enough to stimulate fluorescence but not so high as to structurally damage the target organelle. Power in the 0.5-μm focused spot varied between 92 and 1230 nW.

The laser was focused into a single mitochondrion (see Fig. 1) that had been selected by phase-contrast observation prior to allowing the laser to enter the microscope. A calibrated reticle in the ocular of the microscope was used to identify the precise location of the focused laser beam. Once the calibration point where the laser was focused was identified, an appropriate target mitochondrion was moved under the point, and the laser was permitted entry into the microscope. An epifluorescent beam splitter that reflects below 510 nm and transmits above 520 nm was used to deflect the laser down to a ×100 phase-

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contrast Neofluar objective, where the beam was focused to a 0.5-μm spot diameter. Wavelengths between 520 and 560 nm emitted from the target were passed up to a detection system, which was a sensitive EMI 9862B/100 photomultiplier tube mounted above the microscope and connected to a Tracor Northern model TN 1710 multichannel analzer. By using this device it was possible to record precisely the fluorescent intensity by counting photons emitted from the target specimen over selected time periods. No attempt was made to measure different spectral components over time. The observations were strictly for changes in total fluorescence between 520 and 560 nm, with increasing time. After each fluorescence measurement was made, the laser focal point was visually checked to determine if it was still focused on the exact same point in the mitochondrion. If the cell had moved or the microscope had gone out of focus, the data were discarded.

Electron Microscopy. Cells that had specific mitochondria irradiated and fluorescent spectra recorded were photographed and fixed for electron microscopy by using a solution of 2.5% (wt/vol) glutaraldehyde in heart media. The irradiated cells were next embedded, serial sectioned, and relocated, using procedures developed in this laboratory (10, 11). The same mitochondrion that was irradiated and spectroscopically observed was analyzed ultrastructurally with a Philips 300 electron microscope operating at 80 kV.

RESULTS

General Fluorescence Patterns and Cell Behavior. Both rhodamine 6G and rhodamine 123 selectively bind to the mitochondria (Fig. 1 *A* and *B*) and render these organelles brightly fluorescent (12). This is the case with respect to myocardial cells and endothelial cells. The mitochondria of the myocardial cells are large and spherical, whereas the endothelial cell mitochondria are smaller and filamentous. The spatial fluorescence patterns of the two rhodamines were identical. However, it was observed that under normal mercury lamp-stimulated fluorescence, the rhodamine 6G fluorescence at the same spot seemed to be varying greatly in brightness over a 2- to 5-min period. Although fluorescent patterns of rhodamine 6G-stained cells persisted for up to 48 hr after labeling, the overall intensity of the fluorescence in the rhodamine 123 cells was practically undetectable by 48 hr. Neither of the rhodamine treatments appeared to inhibit cell contractility for up to 48 hr after treatment. Although earlier studies of Gear (1) indicated that continued exposure to rhodamine 6G inhibited mitochondrial oxidative phosphorylation when 7–20 μM solutions were used on isolated mitochondrial preparations, it should be noted that our studies employed intact living heart cells that were stained briefly (30 min) with a dye concentration of less than 3 μM.

Laser-Stimulated Fluorescence. The laser beam was focused to less than 1 μm in diameter and directed into specific regions of single large mitochondria (Fig. 1*C*). Because some of the mitochondria were 3–4 μm in diameter, it was possible to record fluorescence emissions from different regions of the same mitochondrion. This size relationship is depicted in Fig. 1*C*. Fluorescence emissions were recorded as follows: (*i*) from different points in the same mitochondrion, (*ii*) from different mitochondria in the same cell, (*iii*) from different mitochondria in different cells.

The fluorescence emission for rhodamine 123 was essentially a high level of fluorescence, with a slight gradual decay as time progressed (Fig. 2*A*). This was the case for measurements in all three categories listed in the previous paragraph. The fluorescence patterns for rhodamine 6G were much more interesting. Whereas the fluorescent emissions of single mitochondria of endothelial cells were generally constant with perhaps a slight decrease (Fig. 2*B*), the fluorescence patterns of the myocardial mitochondria were of three general types: (*i*) An oscillatory pattern, consisting of a high level of fluorescence with an intense burst of fluorescence (a "flash") followed immediately by a rapid

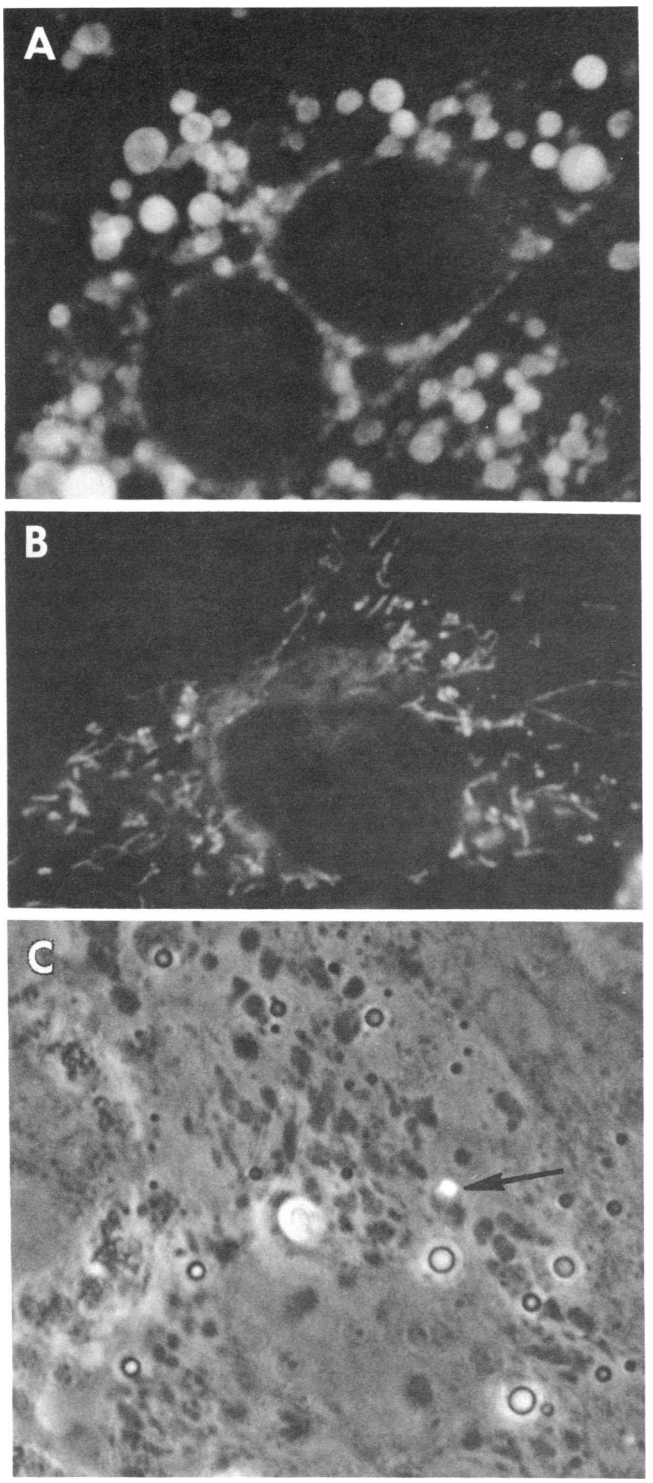

FIG. 1. (*A*) Fluorescence micrograph of rat myocardial cell stained with 1.8 μM rhodamine 6G; note the brightly fluorescent spherical mitochondria. (×2300.) (*B*) Fluorescence micrograph of rat endothelial cell stained as above; note the filiform fluorescing mitochondria. (×2300.) (*C*) Phase-contrast micrograph of myocardial cell with dark mitochondria visible in cytoplasm; laser-stimulated fluorescence spot (arrow) is evident on the corner of one mitochondrion. (×4100.)

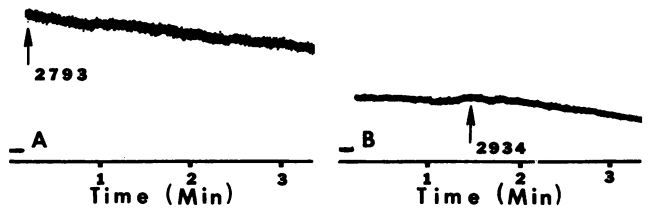

FIG. 2. (*A*) Fluorescence emission over time (520–560 nm) at a fixed point in one rhodamine 123-stained mitochondrion. (*B*) Fluorescence emission over time (520–560 nm) of a rhodamine 6G-stained mitochondrion in an endothelial cell. Counted photons at the arrows are indicated.

and steep decrease in fluorescence; the same point in the mitochondrion would then increase in fluorescence again [sometimes to the same level as before (Fig. 3*A*) and other times not as high as the first level (Fig. 3*B*)] and undergo a flash and a decrease again. This pattern was observed to occur repeatedly at the same spot in an oscillatory fashion for up to several minutes. (*ii*) A high level of fluorescence with many irregular increases and decreases in fluorescence (Fig. 3*C*) but no major decrease of the magnitude observed in the previous cases. (*iii*) A high level of fluorescence followed by a burst (flash) of fluorescence and then a very rapid, steep, decline in fluorescence; the same point in the mitochondrion would continue to fluorescence at a low level with no major increase in fluorescence (as in the preceding situations), though some small bursts of fluorescence were occasionally detected (see Fig. 3 *D* and *E*).

It was interesting to observe that different patterns of fluorescence were detected at different points in the same mitochondrion (see Fig. 3 *B*, *C*, and *F*) or in different mitochondria in the same cell. It was also observed that in some mitochondria only one pattern of fluorescence was detected when measurements were made at different sites. In addition it did not appear to make a difference if the myocardial cell was contracting or quiescent. It was interesting to observe that in a contracting myocardial cell the mitochondrion did move slightly with each contraction and relaxation cycle. There was a corresponding increase and decrease in fluorescence when the laser spot moved either to a different point of the mitochondrion or outside of the mitochondrion (Fig. 3*D*). However, it appeared that with each contraction–relaxation cycle the mitochondrion always returned to the same position because the general pattern of fluorescence was maintained.

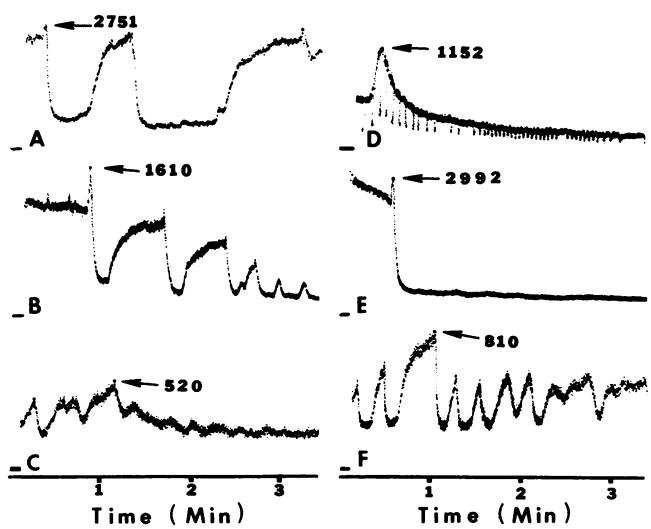

FIG. 3. Rhodamine 6G fluorescence emission at 520–560 nm in 0.5-μm spots in single mitochondria. Curves *B*, *C*, and *F* are from different spots in the same mitochondrion. *A*, *D*, and *E* are from different mitochondria in different cells. *D* is a contracting cell and exhibits a major fluorescence drop each time the cell contracts and the mitochondrion moves out from under the laser beam. The arrows in each part of the figure indicate the maximal number of counted photons. The bar next to each letter indicates zero photon counts just prior to exposure of the mitochondrion to the laser beam.

Electron Microscopy. Mitochondria that were laser-irradiated and fluorescently analyzed were examined with the transmission elecron microscope. Rhodamine 6G itself does have a slight effect on the mitochondria ultrastructure, as evidenced by the formation of some intramitochondrial vesicles and the occasional disruption of the normal lamellar cristae pattern. However, the mitochondria do appear to have large amounts of normal cristae and normal-appearing outer and inner mitochondrial membranes. In addition, the cells are contracting rhythmically at normal rates. Serial sections of a laser-irradiated mitochondrion appear identical to sections of control unirradiated mitochondria (Fig. 4).

DISCUSSION

A method to study the structure and function of organelles at the suborganelle level in a living cell is now available. Because of the coherence, monochromaticity, and single-mode (TEM_{oo}) structure of the laser beam, it is possible to focus it to an intense spot approaching diffraction limits, $\lambda/2$, and therefore monitor fluorescence in localized regions of 0.25–1 μm.

The observations presented here suggest that: (*i*) There are different functional classes of mitochondria in different cell types in the same culture or tissue (as evidenced by the totally different curves obtained for mitochondria in myocardial versus endothelial cells). (*ii*) There are biochemical "microdomains" within a single mitochondrion that can be localized down to suborganelle regions between 0.25 and 1 μm in diameter. The fact that distinctly different fluorescence patterns can be obtained from different points in the same mitochondrion supports the second point. Furthermore, the ability to obtain bright fluorescence followed by a nonrecoverable fluorescence decay in one part of the mitochondrion, and intense oscillating fluorescence in another part of the same mitochondrion, suggests a certain heterogeneity and compartmentalization within the organelle.

It has already been shown (13, 14) that rat liver mitochondria can undergo a marked oscillation in volume as measured by light scattering. The periodic increases in volume (swelling) followed by a rapid decrease and then increase again has been shown to be related to changes in mitochondrial membrane permeability and electron transfer within the mitochondrion. In addition, these same authors demonstrated that oscillatory changes in mitochondrial volume and respiratory rate occurred naturally in mitochondria that were in a state of aerobic respiration. Furthermore, they demonstrated that the average period of an oscillation (volume or respiratory) was 100 sec at 25°C. The oscillatory curves of volume and respiratory changes in mitochondria presented by these authors are remarkably similar to the fluorescent patterns we have described in this paper. In fact, figure 1 in the paper by Mustafa *et al.* (13) contains oscillatory patterns that have small bursts of volume increase just prior to a steep major decrease. These patterns are identical to our fluorescent curves (Fig. 3). Furthermore, the period of the oscillations in those studies and the ones reported here are similar. Because it has already been demonstrated that rhodamine 6G reflects changes in membrane permeability and charge separation (5, 6), the fluorescent oscillations we have reported here are more

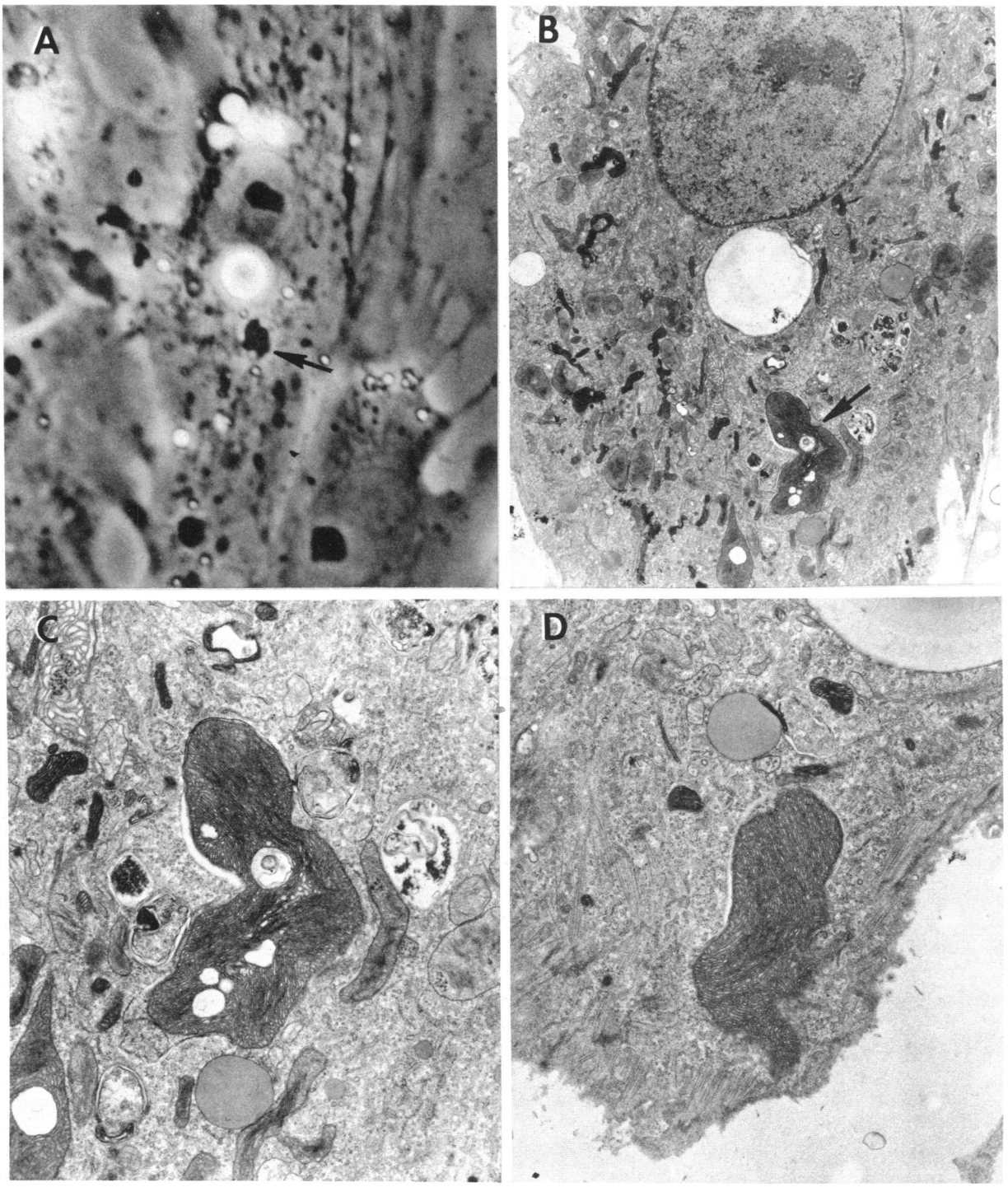

FIG. 4. Light and electron micrographs of a cell that had a specific fluorescence pattern recorded from one mitochondrion. (*A*) Light micrograph illustrating mitochondrion (arrow) from which fluorescence was recorded. (×1400.) (*B*) Low-power electron micrograph of same cell. (×3500.) (*C*) Higher magnification illustrating irradiated mitochondrion; intramitochondrial vesicles are caused by rhodamine treatment, not laser. (×9500.) (*D*) Serial section through irradiated mitochondrion, showing normal ultrastructure. (×9500.)

than likely reflecting localized changes in membrane permeability, ion flux, and associated respiratory events. In light of earlier studies demonstrating mitochondrial oscillation (13, 14) and our Fig. 3*D* demonstrating what an artifactual oscillatory pattern looks like in a moving mitochondrion, the results reported here cannot be artifactual.

Employing specific inhibitors of electron transport in combination with laser-induced fluorescence should illuminate these observations and allow further dissection of suborganelle functions in a living, contracting cell.

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